

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE (DD-MM-YYYY) 09/20/2004			2. REPORT TYPE Final technical report			3. DATES COVERED 11-01-99 - 05-31-01		
4. TITLE AND SUBTITLE Extending Refrigerated Storage of Red Blood Cells						5a. CONTRACT NUMBER		
						5b. GRANT NUMBER N 00014-98-1-0451		
						5c. PROGRAM ELEMENT NUMBER		
						5d. PROJECT NUMBER		
6. AUTHOR(S) Bitensky, Mark W. Yoshida, Tatsuro						5e. TASK NUMBER		
						5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Visual and Circulatory Biophysics Laboratory Rm 311 Department of Biomedical Engineering Boston University 36 Commington St. Boston, MA 02215						8. PERFORMING ORGANIZATION REPORT NUMBER 4184-5		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 800 N. Quincy St. Arlington, VA 22217-5000						10. SPONSOR/MONITOR'S ACRONYM(S) ONR		
						11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution unlimited								
13. SUPPLEMENTARY NOTES								
14. ABSTRACT See accompanying sheet								
15. SUBJECT TERMS Deoxygenated blood storage, Extended shelf life, Oxygen removal, Metabolic supplements, Improved blood quality								
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT		18. NUMBER OF PAGES		19a. NAME OF RESPONSIBLE PERSON	
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unlimited		4		Bitensky, Mark W.	
						19b. TELEPHONE NUMBER (Include area code) 617 353 1637		

20040923 020

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1. REPORT DATE. Full publication date, including day, month, if available. Must cite at least the year and be year 2000 compliant, e.g. 30-06-1998; xx-06-1998; xx-xx-1998.

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ABSTRACT

Oxygen removal increases shelf-life and quality of refrigerated blood. The shelf life of our blood has been prolonged to a minimum of 12 weeks with survival equal to or greater than that of conventional 6 week storage. Increased quality is evident in increased ATP and DPG. This may result from increased binding of ATP and DPG to hemoglobin as a consequence of oxygen removal. The extent of quality improvement and prolonged shelf life are not yet maximal. Strong possibilities exist for further enhancing quality and prolonging shelf life. A self-regulating blood bag compatible with conventional storage facilities has been designed and awaits testing.

**FINAL TECHNICAL REPORT
OFFICE OF NAVAL RESEARCH**

Grant Number: N00014-98-1-0451

Principal Investigator: Mark Bitensky.

Institution: Department of Biomedical Engineering, Boston University.

Grant Title: "Extending the refrigerated storage of red blood cells".

Award Period: 1 November 1999 - 31 May 2001.

Objective: To achieve an improvement in the logistics of blood transfusion as a result of shelf life extension accompanied by enhanced quality. We suggest that without the emphasis on improved quality, shelf life extension represents a hollow victory. When, however, shelf life extension is accompanied by improved quality, the dual accomplishment provides a better blood product with improved logistics.

During conventional storage the quality of blood significantly deteriorates so that by the end of 6 weeks it is not longer suitable for clinical use (the established criteria in transfusion practice is a minimum of 75% of the cells demonstrating 24 hour *in vivo* survivals). Since conventional storage yields a product which is far more perishable than is desired, it is clear innovations in storage which can improve both shelf life and quality will materially benefit the current supply inadequacies. We place strong emphasis on the fact that 2,3 DPG is unmeasurable after less than one week of conventional storage. This defect in quality is one of the important difficulties addressed by our research program.

Approach: Our efforts provide yet another example of an experimental program graced by serendipity. The approach we took worked wonderfully well, but not for the reasons we thought it would.

At the outset, our research team (henceforth referred to as "we") examined the existing information on the evolution of the cellular and metabolic deficiencies that were associated with conventional blood storage. The most outstanding feature of storage-related changes was the progressive loss of red cell membrane area through the process of vesicle production. It is now a documented experimental fact that hemoglobin (Hb) denaturation drives vesiculation-based membrane loss. In association with denaturation, Hb molecules expose concealed hydrophobic domains and therefore partition into the red blood cell membrane bilayer. They are marked by the persistence of phosphatidyl serine (PS) on the outer surface of the membrane. In short, the process of ineluctable Hb denaturation is the driver/timekeeper of the membrane loss that accompanies vesicle production. Splenic macrophages continually excise those microscopic domains of the red cell membrane in which small islands of exposed PS coalesce. Such membrane domains either spontaneously vesiculate or are effectively excised by splenic macrophages. This process of Hb denaturation-driven red blood cell membrane vesicle production occurs during refrigerated storage at 4° C. In effect, the rate of membrane loss or vesicle shedding by refrigerated RBC is driven by and is an expression of the rate of Hb denaturation.

Our study was initiated with the intention of slowing or retarding the Hb denaturation rate. Early in our work we did observe that when oxygen was removed as a component of refrigerated red cell storage, red cell membrane area loss slowed down. In retrospect, we have come to understand that while this result was both significant and beneficial, it was nevertheless a serendipitous result. That is, the actual process causing membrane loss at 4°C was somewhat different from the process we originally envisioned.

We initially believed that Hb denaturation and membrane area loss were dependent on oxygen, i.e., oxygen was driving the denaturation process and its removal was beneficial because oxygen was essential for the oxidative consumption of red blood cell membrane. We have since come to understand that the dramatic benefits that appear in consequence of oxygen removal are actually explained as follows. The deoxygenated red blood cell offers a large, stable reservoir of Hb-based nucleotide binding sites, including sites for ATP and DPG. Thus, in deoxygenated blood, Hb can bind and stabilize both nucleotides. In turn, deoxygenated Hb, by binding ATP and DPG, is stabilized in a conformation that is resistant to denaturation. This then reduces the rates at which RBC membrane is lost. Deoxygenation of blood is reliably associated with a marked improvement in RBC quality and an extension of RBC shelf life. The improvement in RBC quality is best understood in terms of sustained levels of ATP and DPG. Thus, Hb denaturation and membrane loss are simultaneously reduced, and when deoxygenated RBC are stored at 4° one observes improved membrane area retention, and improved levels of ATP and DPG. The serendipitous consequence of oxygen removal is largely a result of Hb stabilization by red blood cell nucleotides rather than a reduction in the rates of oxidation dependent Hb damage.

The additional benefit of oxygen removal resulting from reduced oxidation rates is in fact miniscule. We suggest that at 4°C, oxidative reactions are extremely slow and do not serve as a significant source of Hb damage. We also note that at 37° *in vivo*, when oxygen is present at saturating levels at the same time that ATP and DPG levels are maximal, the rates of Hb denaturation are extremely slow in comparison with those rates which were observed when glycolysis was inhibited in the absence of oxygen. In such circumstances Hb denaturation reflected reduced nucleotide concentrations rather than oxygen associated damage. (Data not shown.)

Accomplishments.

The actual accomplishments of our research program are summarized in Table I. It is noteworthy that a 6 subject cohort achieved twenty-four hour survival values of 76.6% after 12 weeks of storage at 4°C. Of equal importance are the following data.

- Percent hemolysis was only 0.5.
- 2,3 DPG levels were 9.37 μ mol/g Hb.
- ATP levels were 5.03 μ mol/g Hb.

These are the data that demonstrate the simultaneous achievement of shelf life extension accompanied by markedly improved quality.

The composition of the additive solution used in these experiments is given in Table II.

TABLE I

***IN VIVO* SURVIVAL STUDIES: COMPARISON OF CONVENTIONAL AND HEMANEXT BLOOD**

Storage duration /additive solution	No. of subjects	24-hr <i>in vivo</i> survival (%)	% Hemolysis	2,3-DPG $\mu\text{mol/g}$ Hb	ATP $\mu\text{mol/g}$ Hb
Fresh blood	7*	-	0.08	10.74	4.3
6 wks/conventional	>50	75-80	0.5	0.5	3.5
6 wks/Hemanext	>24	>90	0.4	9.0-12.0	4.8
10 wks/conventional	7*	67.1	0.56	0.75	2.82
10 wks/ Hemanext	7*	83.2	0.41	9.41	5.08
12 wks/ Hemanext	6*	76.6	0.5	9.37	5.03
14 wks/ Hemanext	6*	70	0.56	11.06	4.41
14 wks/ Hemanext	3 of 6*	78.1	0.77	10.57	4.12
16 wks/ Hemanext	2 of 6*	76.7	1.15	10.82	3.56

The data shown are for units that were not leuko-reduced.

*These data are from a single patient cohort (tested at Dartmouth Hitchcock).

TABLE II

Supplement Concentrations

Parameters	Additives (mM)
[ATP] and [2,3-DPG] (Valeri)	adenine (0.5) Pi (0.73) glucose (60) inosine (10) pyruvate (10)
[GSH] (Dumaswalla)	N-acetyl-cysteine (0.25) glutamine (0.25) glycine (0.25)

Conclusions. Oxygen removal in combination with a simple additive solution achieves a doubling of useful storage life accompanied by substantial and measurable improvements in blood quality.

Significance. We suggest that the results achieved with deoxygenated RBC storage represent an important advance in the logistics and quality of the blood storage product. Since we did not have an opportunity to optimize each of the storage parameters, it is likely that further improvements in shelf life and quality are possible using this general approach. The improved blood product seems especially relevant for combat casualties requiring multiple transfusions and as well for autologous transfusions

and pediatric surgery. It is noteworthy that experience has elicited the practice among pediatric surgeons of selecting units of blood that have been in refrigerated storage only 1 or 2 weeks.

Patent Information.

1. Case Application No. 60/332405

Filed: Nov. 16, 2001

"Additive solution for blood preservation".

2. Case application No. 60/332409

Filed Nov. 16, 2001

"Addition of metabolic supplementaiton during refrigerated, oxygen-depleted red cell storage".

Award Information. Principal investigator remains Research Professor and Director of Visual and Circulatory Biophysics Laboratory.